

NON-PROVISIONAL
UTILITY PATENT APPLICATION
TRANSMITTAL - 37 CFR 1.53(b)

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Assistant Commissioner for Patent
BOX PATENT APPLICATION
Washington, DC 20231

Attorney Docket No. 7600-51U1 (CHOP-0088)
First Named Inventor: Katherine A. High *et al.*
Express Mail Label No. EL471633245US
Total Pages of Transmittal Form: 2



Transmitted herewith for filing is the non-provisional utility patent application entitled:

**METHODS FOR PREVENTING FORMATION OF INHIBITORY ANTIBODIES
IN THE SETTING OF GENE THERAPY**

which is:

an ☒ Original; or

a ☐ Continuation, ☐ Divisional, or ☐ Continuation-in-part (CIP)
of prior Application No. ____/____, filed ____.

☒ This non-provisional patent application is based on Provisional Patent Application
No. 60/138,066, filed June 8, 1999.

Enclosed are:

- ☒ Specification (including Abstract) and claims: 19 pages.
- ☒ Non-executed copy of Declaration.
- ☐ Copy of Declaration from prior application.
- ☐ Separate Power of Attorney (including 37 CFR 3.73(b) statement, if applicable).
- ☐ sheets of drawings (formal) plus one copy.
- ☐ Microfiche computer program (Appendix).
- ☐ Nucleotide and/or Amino Acid Sequence Submission, including:
 - ☐ Computer readable copy ☐ Paper Copy ☐ Verified Statement.
- ☐ Under PTO-1595 cover sheet, an assignment of the invention.
 - ☐ Certified copy of _____ Application No. _____, filed _____, is filed:
☐ herewith or ☐ in prior application _____.
- ☒ Verified Statement Claiming Small Entity Status under 37 CFR 1.9 and 1.27.
 - ☐ was filed in the prior non-provisional application, and such
status is still proper and desired (37 CFR 1.28(a));
 - ☒ is enclosed herewith; ☐ is no longer desired.
- ☐ Preliminary Amendment.
- ☐ Information Disclosure Statement, PTO-1449, and cited references.

The filing fee has been calculated as shown below:

			SMALL ENTITY			LARGE ENTITY	
CLAIMS	NO. FILED	NO. EXTRA	BASIC FEE:			BASIC FEE:	
			\$345			\$690	
Total	12-20 =	0	X9	\$.00	OR	X18	\$
Independent	1-3 =		X39	\$	OR	X78	\$
Multiple Dependent Claims Present: &@			\$130	\$	OR	\$260	\$
			TOTAL	\$-0-*	OR	TOTAL	\$

*The above calculation fee is not being paid at this time.

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- ☐ Any additional fees required under 37 C.F.R. §1.16.
- ☒ Any additional fees required under 37 C.F.R. §1.17.
- ☒ If the filing of any paper during the prosecution of this application requires an extension of time in order for the paper to be timely filed, applicant(s) hereby petition(s) for the appropriate extension of time pursuant to 37 C.F.R. §1.136(a).

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Enclosures

Applicant or Patentee: Katherine High *et al.*
Application or Patent No.: Not Yet Assigned
Filed or Issued: June 8, 2000
For: **METHODS FOR PREVENTING FORMATION OF
INHIBITORY ANTIBODIES IN THE SETTING OF GENE
THERAPY**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION**

NAME OF ORGANIZATION: The Children's Hospital of Philadelphia
ADDRESS OF ORGANIZATION: 34th & Civic Center Boulevard
Philadelphia, Pennsylvania 19104-4318

TYPE OF ORGANIZATION:

- ☐ University or other institution of higher education.
☒ Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)).
☐ Nonprofit scientific or educational under statute of state of the United States of America.
Name of State _____
Citation of Statute _____
☐ Would qualify as tax exempt under Internal Revenue Code (26 USC 501(a) and 501(c)(3) if located in the United States of America.
☐ Would qualify as nonprofit scientific or educational under statute of state of United States of America if located in the United States of America.
Name of State _____
Citation of Statute _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention of the above-identified patent or patent application.

I hereby declare that U.S. rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME _____
ADDRESS _____

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in the status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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METHODS FOR PREVENTING FORMATION OF INHIBITORY ANTIBODIES IN THE SETTING OF GENE THERAPY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority under 35 U.S.C. §119(e) to U.S.
5 Provisional Application No. 60/138,066, filed on June 8, 1999.

STATEMENT OF FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made in part using funds obtained from the U.S.
10 Government (National Institutes of Health Grant No. R01 HL61921) and the U.S.
Government may therefore have certain rights in the invention.

BACKGROUND OF THE INVENTION

The process of blood coagulation involves a series of proteins known as
15 blood coagulation proteins which act in a cascade fashion to effect the formation of a
blood clot. Hemophilia is a disease of humans and other mammals wherein a gene
encoding a blood coagulation factor contains a mutation such that the encoded protein
does not function normally in the cascade process. Specifically, the hereditary disease,
hemophilia B, is characterized by a mutation in the gene encoding the blood
20 coagulation protein, Factor IX (F.IX). F.IX is reviewed in High *et al.* (1995, "Factor
IX" In: Molecular Basis of Thrombosis and Hemostasis, High and Roberts, eds.,
Marcel Dekker, Inc.).

Adenoviral vectors are well known in gene therapy and have been used
to effect expression of high levels of canine factor IX in immunodeficient mice or in
25 immunocompetent mice when the virus is administered in conjunction with
immunosuppressive agents. When adenoviral vectors are administered to
immunocompetent mice in the absence of immunosuppressive agents, these vectors
induce a strong inflammatory and cytotoxic T lymphocyte (CTL) response (Dai *et al.*,

1995, Proc. Natl. Acad. Sci. USA 92:1401-1405) which negates the beneficial effects of the therapy. In addition, there are reports which suggest that intramuscular injection of replication defective adenovirus provides long-term expression of a transgene, provided that the transgene encodes a self-protein (*i.e.*, a host protein), such that a strong host immune response is avoided (Tripathy et al., 1996, Nature Med. 2:545-550; Yang et al., 1996, Hum. Mol. Genet. 5:1703-1712). Thus, while there has been significant progress in the area of gene therapy in *in vivo* expression of a selected transgene following direct injection of an adenoviral vector into skeletal muscle, the use of adenoviral vectors may not be the optimal method for gene therapy in light of these immunological considerations.

Retroviral vectors have also been used experimentally as a model for treatment of hemophilia B. However, levels of expression of F.IX from these vectors are reported to be too low to be of therapeutic value (Kay et al., 1993, Science 262:117-119).

Plasmid DNA which has been injected into mouse muscle has been shown to direct expression of erythropoietin (Epo) (Tripathy et al., 1996, Proc. Natl. Acad. Sci. USA 93:10876-10880), but this method of gene therapy is apparently not sufficiently efficient for the expression of a gene product such as F.IX, which is needed at relatively high levels in the circulation (compared with Epo) to achieve a therapeutic effect.

Adeno-associated virus (AAV) is an alternative vehicle to adenovirus for delivery of genes to muscle. Recombinant AAV (rAAV) does not contain sequences encoding viral proteins and has the potential to integrate into the chromosomal DNA of the host cell (Carter, 1992, Curr. Opin. Biotech. 3:533-539; Skulimowski et al., 1995, Method Mol. Genet. 7:7-12). Production and purification procedures are now available which facilitate the generation of pure rAAV which is not contaminated by wild-type AAV or helper adenovirus. As noted herein, administration of adenovirus to mammals is accompanied by the aforementioned immunological problems.

While the efficiency of in vivo transduction with rAAV in the absence of helper virus is low for some cell types, certain post-mitotic cells such as neurons (Kaplit et al., 1994, Nature Genet. 8:148-154) and skeletal muscle fibers (Xiao et al., J. Virol. 70:8098-8108) can be effectively transduced with this vector. Stable expression of lacZ for up to 1.5 years has been reported (Xiao et al., supra). In contrast to adenoviral vectors, intramuscular injection with rAAV in immunocompetent animals does not result in a CTL response against transduced muscle fibers, nor are circulating antibodies against the intracellular lacZ gene product present.

The results of Southern blot hybridization and PCR analyses of genomic DNA obtained from skeletal muscle injected with rAAV establish that rAAV genomes persist in the form of tandem repeats which may be integrated into chromosomal DNA (Xiao et al., supra). The expression of the secreted protein, Epo, following intramuscular injection with rAAV is reported in Kessler et al. (1996, Proc. Natl. Acad. Sci. USA 93:14082-14087). However, the levels of protein expression reported were one to two orders of magnitude below that required for a therapeutic effect mediated by F.IX.

Current therapy for hemophilia involves the intravenous injection of a preparation of clotting factor concentrates whenever a bleed occurs. This treatment is cumbersome, inconvenient and very expensive. The average patient pays approximately \$100,000 per year for the concentrate alone. Further, because the concentrate is only administered to the patient intermittently, patients remain at risk for life-threatening bleeds which are fatal if treatment is not timely administered.

In a gene therapy based setting, the production of a secretable protein in a host animal, wherein there is no endogenous expression of the gene in the host animal, may lead to an immune response to the secreted gene. While a successful regimen of gene therapy has been disclosed in International Application PCT/US98/04790, there is a need for the development of improvements to the procedures described therein. Further, there is a long felt and acute need for improved

methods of delivering F.IX to mammals, in particular, to humans, having hemophilia such that a therapeutic effect is achieved. The present invention satisfies this need.

SUMMARY OF THE INVENTION

5 The invention relates to a method of preventing the formation of inhibitory antibodies in a mammal undergoing gene therapy. The method comprises administering to the mammal an immunosuppressive agent in conjunction with the gene therapy.

In one aspect, the mammal is a human.

10 In another aspect, the gene therapy is delivery of a nucleic acid to the mammal, which when expressed in the mammal, serves to correct a genetic defect in the mammal.

In yet another aspect, the protein is selected from the group consisting of Factor VII, Factor VIII, Factor IX, Factor X, alpha1-antitrypsinogen, glucuronidase, a sarcoglycan, an interferon, insulin-like growth factor, and erythropoietin.

15 In a preferred embodiment, the gene therapy is delivery of Factor IX to the mammal.

In yet a further aspect, the gene therapy is performed by administering a viral vector to the mammal, wherein the viral vector comprises a nucleic acid to be delivered to the human. Preferably, the viral vector is an adeno-associated viral vector.

20 Also preferably, the Factor IX is delivered to the mammal using an adeno-associated virus vector.

In another aspect, the immunosuppressive agent is selected from the group consisting of cyclophosphamide, FK506, anti-CD40 ligand, CTLA4Ig, cyclosporin, antiB71-B72, and an immunosuppressive steroid.

25 In a preferred embodiment, the immunosuppressive agent is cyclophosphamide.

In another preferred embodiment, the immunosuppressive agent is FK506.

In a further preferred embodiment, the mammal has hemophilia B and the inhibitory antibodies specifically bind with Factor IX protein.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is a diagram demonstrating sites of action of various immunomodulatory agents within the immune system. APC stands for antigen presenting cell.

 Figure 2, comprising Figures 2A and 2B, is a series of graphs illustrating the data obtained when the mice received anti-CD40 ligand (anti-CD40L) as well as AAV-mF.IX. Figure 2A depicts aPTT values and indicates that the mice never exhibited correction of the aPTT. Figure 2B depicts Bethesda titers and indicates that inhibitory antibodies were still present even when the anti-CD40 ligand was co-administered to the animals.

10 Figure 3 is a graph depicting aPTT values obtained from mice treated with cyclophosphamide at the time of AAV-mF.IX vector administration.

 Figure 4, comprising Figures 4A-4D, is a series of images of the results of Western blotting experiments depicting antibody to murine F.IX in hemophilic mice injected with AAV-mF.IX.

15 Figure 5 is a graph illustrating data which establish that the immunosuppressive agent, FK506, is useful for the prevention of formation of inhibitory antibodies, in that, mice treated with FK506 exhibited shorter aPTT coagulation times than mice which were not treated with FK506.

DETAILED DESCRIPTION OF THE INVENTION

20 The invention relates to the discovery of a method of preventing the formation of inhibitory antibodies to blood clotting proteins in a mammal administered the proteins in a gene therapy protocol. The most serious complication of protein-based therapy for hemophilia is the formation of inhibitory antibodies to blood clotting factors. These antibodies are also induced in the context of gene based therapy for hemophilia. The invention provides a method of preventing the formation of inhibitory

antibodies in the setting of gene therapy for hemophilia, wherein the method comprises administration to a mammal undergoing gene therapy an immunosuppressive agent to the mammal.

The gene therapy methods for which the present invention is applicable are described in International Application PCT/US98/04790 which is hereby incorporated herein by reference in its entirety. As such these methods are not exhaustively repeated in the present application. Moreover, such technology is now well known in the art being described, for example, in Herzog et al., 1997, Proc. Natl. Acad. Sci. USA 94: 5804-5809; Herzog et al., 1999, Nature Medicine 5:56-63; Herzog and High, 1999, AAV-mediated gene transfer of factor IX for treatment of hemophilia B by gene therapy. In: Thrombosis and Haemostasis, 1999 State of the Art, Hoyer L (ed.), 82:540-546. Matsushita et al., 1998, Gene Ther. 5:938-945; Matsushita et al., 1999, Improvements in AAV vector production: Elimination of pseudo-wild type AAV. Proceedings of the 2nd annual American Society of Gene Therapy, Washington, DC, June 9-13, p. 2a; Nakai et al., 1998, Blood 91:4600-4607.

Definitions

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element. APlurality \equiv means at least two.

As used herein, "treating a disease" means reducing the frequency with which a symptom of the disease is experienced by a patient.

The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen.

Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules.

By the term "inhibitory antibody" as used herein is meant an antibody which specifically binds to a protein expressed in a mammal, which protein has been delivered to the mammal by way of gene therapy.

By the term "specifically binds," as used herein, is meant an antibody which recognizes and binds a specific protein in a sample, but does not substantially recognize or bind other molecules in the sample.

A "vector" is a composition of matter which comprises an isolated nucleic acid and which can be used to deliver the isolated nucleic acid to the interior of a cell. Numerous vectors are known in the art including, but not limited to, linear polynucleotides, polynucleotides associated with ionic or amphiphilic compounds, plasmids, and viruses. Thus, the term "vector" includes an autonomously replicating plasmid or a virus. The term should also be construed to include non-plasmid and non-viral compounds which facilitate transfer of nucleic acid into cells, such as, for example, polylysine compounds, liposomes, and the like. Examples of viral vectors include, but are not limited to, adenoviral vectors, adeno-associated virus vectors, retroviral vectors, and the like.

"Expression vector" refers to a vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis-acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate the recombinant polynucleotide.

"Immunosuppressive agent," refers to a compound which is capable of suppressing the immune response in a mammal. Preferably, the immunosuppressive agent exerts an effect in the mammal which results in a reduced ability of the mammal to generate antibodies directed against a desired protein, specifically a protein delivered to the mammal in a gene therapy protocol.

“Gene therapy,” refers to the process of correcting a genetic defect in a mammal comprising administering to the mammal a nucleic acid, which when expressed in the mammal, produces a protein which serves to correct the defect. The process also refers to the administration of a nucleic acid to a mammal where the administered nucleic acid serves to correct a genetic defect in the mammal whether or not a protein is expressed by the administered nucleic acid, i.e., for example, the process of DNA repair.

A “gene therapy based protocol” is a protocol in which gene therapy is accomplished.

The Invention

The invention includes a method of preventing the formation of inhibitory antibodies in a mammal undergoing gene therapy wherein the method comprises administering to the mammal an immunosuppressive agent in conjunction with the gene therapy. As noted elsewhere herein, gene therapy typically involves delivery of a vector to a mammal, wherein the vector comprises an expressible DNA encoding a desired protein. When the vector is delivered to the mammal and the protein is expressed therein, a beneficial effect is experienced by the mammal. However, in certain instances, the immune system of the mammal reacts to the presence of the expressed protein and mounts an antibody response thereto through the production of an inhibitory antibody. Thus, with time, or even immediately, the gene therapy is rendered ineffective because the inhibitory antibody specifically binds to the delivered protein rendering the protein incapable of exerting the desired beneficial effect to the mammal. Administration of the immunosuppressive agent to the mammal serves to diminish or ablate the generation of inhibitory antibodies which specifically bind to the protein so delivered.

It is not necessary in the method of the invention that the immunosuppressive agent completely block the production of inhibitory antibodies in the mammal. Rather, in some instances it may be sufficient that the immunosuppressive agent simply reduce the amount of antibody which would

normally be produced in the absence of administration of the immunosuppressive agent.

While preferred embodiments of the invention are described herein, it is important to note that the invention should not be construed to be limited to these
5 embodiments. Rather, the invention should be construed to include the use of an immunosuppressive agent in any type of gene therapy.

In one preferred embodiment, the mammal is a human.

In another preferred embodiment, the gene therapy vector is AAV. However, the invention should not be construed as being limited to this vector. Rather,
10 other suitable vectors which are known in the art, or are as yet unknown, may be useful in the methods of the invention.

In yet another preferred embodiment, the mammal is administered Factor IX (F.IX) in a gene therapy based protocol. However, the invention should not be construed to be limited to gene therapy using F.IX. Rather, the invention should be
15 construed to include the delivery of any protein to a mammal via a gene therapy based protocol. Examples of proteins suitable for delivery include, but are not limited to, any other blood coagulation protein, including, but not limited to, Factor VII, Factor VIII, Factor X, proteins such as alpha1-antitrypsinogen, glucuronidase, sarcoglycans, interferons, insulin-like growth factor, and erythropoietins.

In the method of the invention, the immunosuppressive agent is administered to the mammal prior to, and/or concomitantly with, and/or following administration of the gene therapy vector to the mammal. Depending on the type of gene therapy and the type of immunosuppressive agent being used, it may be necessary to vary the time at which the immunosuppressive agent is administered to the mammal
20 relative to the administration of the therapy itself. One of skill in the art of gene therapy and immunosuppression will know, based on the present disclosure, the state of the art, the type of therapy and immunosuppressive agent and the status of the mammal, how to design a successful protocol with undue experimentation.

A preferred immunosuppressive agent in the present invention is cyclophosphamide. However, the invention should not be construed to be limited solely to the use of this immunosuppressive agent. Rather, the invention should be construed to include any and all suitable immunosuppressive agents which are presently known or which become known. Examples of currently known immunosuppressive agents suitable for use in the present invention include, without limitation, cyclophosphamide, FK506, anti-CD40 ligand, CTLA4Ig, cyclosporin, antiB71-B72, and immunosuppressive steroids.

In a preferred embodiment, and when the gene therapy vector comprises an AAV vector containing DNA encoding F.IX, the immunosuppressive agent is cyclophosphamide.

In another preferred embodiment, the mammal is concomitantly administered about four doses of cyclophosphamide. As disclosed elsewhere herein, such an administration protocol results in the prevention of formation of inhibitory antibodies against F.IX in mice having hemophilia B. The type of hemophilia in the mice used in the present study is the result of the fact that the genome of the mice comprises a large deletion in the gene encoding F.IX; such a genetic defect generally results in a high likelihood of inhibitor formation. Co-administration of cyclophosphamide with a gene therapy vector comprising the F.IX gene, greatly lessened or abolished this undesirable side effect.

Kay et al. (1997, Proc. Natl. Acad. Sci. USA 94:4586-4691) have described the use of other immunomodulatory agents to block the immune response to a gene therapy vector; however, this disclosure does not teach the use of immunosuppressive agents for the purpose of inhibiting antibody development directed against the delivered protein. The present invention is specifically directed to inhibition of the generation of antibody specific for the therapeutic gene product itself. The use of immunosuppressive regimens to prevent the formation of inhibitors once they have occurred in the setting of protein-based therapy has been described. Results have been mixed and this treatment modality is not universally used.

The present invention provides a unique solution to the problem of inhibitor formation which is directed against the gene therapy based delivered protein because it works in the setting of gene therapy; and the success rate of the method is high, even when the genetic defect being corrected (large gene deletion) is most likely to result in the generation of inhibitory antibodies directed against the delivered protein. Thus, the method of the present invention solves the most critical problem in gene therapy for hemophilia, i.e. it prevents the formation of inhibitory antibodies.

The preferred method of the invention comprises the administration of cyclophosphamide concomitantly with the administration of a gene therapy vector for the purpose of alleviating hemophilia. Cyclophosphamide is given in about four doses each having a concentration of about 50 mg/kg of body weight per mammal. The compound may be administered intravenously or intraperitoneally. Alternatively, an equivalent per oral administration (PO) dose can be given. However, other dosages and routes of administration are also contemplated, in that, cyclophosphamide may be administered at a lower dose of, for example, 20 mg/kg of body weight, and/or fewer doses may be administered to the mammal. The amount of dosage will depend on the type of mammal being treated and on the level of gene therapy required to alleviate the disease state in the mammal.

The amount of dosage of any immunosuppressive agent can be determined empirically by the skilled artisan, and will depend on any number of factors known to the artisan, including, but not limited to, the factors mentioned elsewhere herein, for example, the type of immunosuppressive agent used, the nature of the disease being treated, the type of vector and protein used, the overall health of the mammal, gender, the age of the mammal, etc. It is sufficient for the present disclosure that examples of successful protocols be provided to guide the artisan.

When the protocol involves the administration of four doses of cyclophosphamide, and the gene therapy being performed is delivery of an AAV vector comprising F.IX DNA, the first dose of cyclophosphamide is administered within twenty four hours of treatment with the gene therapy vector, and subsequent doses are

administered at two week intervals. In the experiments disclosed herein in the Example, in mice that received this regimen, 3/3 animals exhibited no evidence of inhibitor antibody formation during the period post-administration of the gene therapy vector for as long as at least five months. These data are summarized in detail in the experimental details provided herein.

As noted previously, the invention should in no way be construed as being limited solely to the use of cyclophosphamide, or to the dosages, routes of administration and dose regimens disclosed herein. Rather, the invention should be construed to encompass the use of immunosuppressive agents in conjunction with the administration of a gene therapy vector, wherein the vector is administered to the mammal for the purpose of delivering a protein to a mammal, and the immunosuppressive agent is administered to the mammal for the purpose of preventing the formation of inhibitory antibodies to the delivered protein.

The invention is thus useful for the prevention of inhibitory antibody formation in mammals, including humans, undergoing gene therapy treatment for hemophilia. The major therapeutic benefit of the method of the invention is the prevention of formation of inhibitory antibodies to the transgene product, i.e., to the delivered protein, exemplified herein as a coagulation factor. In addition, transient immunosuppression at the time of vector administration may allow re-administration of the vector to the mammal thereby achieving a higher level of efficacy than would be possible without concomitant transient immunosuppression.

The invention is now described with reference to the following experimental examples. These examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Examples

In a gene based setting the production of a secretable protein in a host animal that has no endogenous expression of the gene may lead to an immune response

to the transgene product. In patients with severe hemophilia B inhibitor formation in response to infusion of coagulation Factor IX (F.IX) concentrate occurs in about 4% of patients. One of the main determinants of inhibitor formation is the genotype of the underlying mutation; in general the greater the loss of coding information the more likely the development of an inhibitory antibody. The risk of inhibitor development is not well understood in the context of a gene therapy approach to hemophilia B.

In the experiments described herein, the prevalence of inhibitor development in a murine model of hemophilia which is caused by a large gene deletion affecting the promoter region and exons 1-3 of the F.IX gene (Blood 90:3962) was assessed. This results in an absence of F.IX transcript and protein. The second aim of the study was to evaluate the effect of immunomodulation in reducing the risk of inhibitor development.

A cohort of six mice were injected intramuscularly with an adeno-associated virus (AAV) vector encoding the murine F.IX transgene (total dose 1×10^{11} vector genomes/mouse). Mice were bled at monthly intervals via tail vein transection, and clotting times of plasma were measured by aPTT assay. None of the mice exhibited correction of the aPTT (59.9-709s; normal values 22-39 second) compared with pretreatment values (53.6-66.9s). Bethesda titers revealed evidence of inhibitory activity in all mice by one month which rose on further testing at two months (see Table below). Antibodies were also detectable on Western Blot, although there was individual variation in temporal appearance and titer. Two other cohorts of mice received immunomodulation around the time of vector administration in an effort to suppress inhibitor formation. One cohort ($n = 4$) received anti-CD40L antibody at a total dose of 0.4 mg per mouse given in four divided doses (0.1 mg at days -3, 0, 3, 6) while the other cohort received cyclophosphamide (CYP, $n = 4$) at doses of 50 mg/kg per mouse biweekly at four time points. CD40L antibody-treated mice exhibited partial correction of the aPTT (46.4-57.4s). In the CYP-treated mice, nearly complete correction of the aPTT was observed (28.9-30.9s) in all mice at one month and this has been maintained for the study period of greater than ten months. These data correlate

with reduction or absence of inhibitor formation in immunosuppressed animals. This study provides evidence of successful suppression of inhibitor formation with immune modulation in a muscle-directed gene therapy for treatment of hemophilia B.

5

Table: Bethesda titer of AAV-F.IX treated mice

Months	1	2
no immunosuppression	2.8//0.9/3/3.5	4.3/16/16/2.4
CYP	0/0/0	0/0/0
anti-CD40L	0/1.3/0/0	0/0/7

The methods used in these experiments include the following:

10

Hemophilia B knockout mice were injected intramuscularly with AAV mF.IX (1×10^{11}) at day 0, without or with immunomodulation therapy. The mice were bled at monthly time points in citrate. The assays which were performed included a clotting based - apTT (measure of correction of hemostatic defect) assay and Bethesda titer (1 BU defined as 50% residual FIX in a 2 hour 50:50 mix). In addition, antibody assays, Western blot analysis and ELISA assays were performed.

15

The immunomodulatory strategies used to address formation of antibodies to the transgene product were as follows:

CD40L antibody - 0.1 mg intraperitoneally at day -3, day 0, day +3, day + 6, day + 9;

Cyclosporin A - 100 mg/kg intraperitoneally three times weekly;

Cyclophosphamide - 50 mg/kg intraperitoneally at day 0, week 2, week 4, week 6;

20

FK506 - 125 μ g subcutaneously on alternate days from day 0.

The Results of the experiments presented in this example are now described in conjunction with Figures which illustrate these results.

Figure 1 is a diagram demonstrating sites of action of various immunomodulatory agents within the immune system. APC stands for antigen presenting cell.

25

Control mice were injected with AAV-mF.IX at day 0. aPTT values were measured and graphed as a function of time after injection. The aPTTs remained constant throughout the duration of the experiment, indicating that no correction of clotting time had occurred. The Bethesda titers, a measure of inhibitory antibody titer, were also assessed as a function of time. These values were high throughout the course of the experiment.

In Figure 2 there is shown the data obtained when the mice received anti-CD40L as well as AAV-mF.IX. The panel on the left comprises aPTT values and indicates that the mice never exhibited correction of the aPTT. The panel on the right comprises Bethesda titers and indicates that inhibitory antibodies were still present even when the anti-CD40 ligand was co-administered to the animals.

In Figure 3 there is shown aPTT values obtained from mice treated with cyclophosphamide at the time of AAV-mF.IX vector administration. In contrast to the previous data, these mice exhibited sustained correction of the aPTT, indicating that inhibitory antibodies never formed in these animals. This remained the case at five months after the original vector injection and fourteen weeks after the last doses of cyclophosphamide was administered.

In Figure 4 there is shown the results of a Western blot depicting antibody to murine F.IX in hemophilic mice injected with AAV-mF.IX. Both the control mice and the anti-CD40 ligand treated mice developed antibodies one month after vector injection, but the cyclophosphamide treated mice did not develop antibodies through the five months of observation.

In Figure 5 there is shown data which establish that the immunosuppressive agent, FK506, is useful for the prevention of formation of inhibitory antibodies, in that, mice treated with FK506 exhibited shorter aPTT coagulation times than mice which were not treated with FK506.

The data presented herein therefore establish that in a murine knockout model of hemophilia B (large gene deletion) all mice injected with AAV mFIX made antibodies which are inhibitory to the transgene product. The antibodies are of a IgG 1

subclass which is in keeping with a T_{H2} response. Antibody formation to the transgene product may be decreased by transient immune suppression at the time of vector administration resulting in correction (or partial correction) of the hemostatic defect.

5 The immunosuppressive agents, cyclophosphamide and FK506, are thus useful for inhibition of the generation of inhibitory antibodies directed against a desired protein in mammals receiving gene therapy.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

10 While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of preventing the formation of inhibitory antibodies in a mammal undergoing gene therapy, said method comprising administering to said mammal an immunosuppressive agent in conjunction with said gene therapy.
2. The method of claim 1, wherein said mammal is a human.
3. The method of claim 1, wherein said gene therapy is delivery of a nucleic acid to said mammal, which when expressed in said mammal, serves to correct a genetic defect in said mammal.
4. The method of claim 3, wherein said protein is selected from the group consisting of Factor VII, Factor VIII, Factor IX, Factor X, alpha1-antitrypsinogen, glucuronidase, a sarcoglycan, an interferon, insulin-like growth factor, and erythropoietin.
5. The method of claim 4, wherein said gene therapy is delivery of Factor IX to said mammal.
6. The method of claim 1, wherein said gene therapy is performed by administering a viral vector to said mammal, wherein said viral vector comprises a nucleic acid to be delivered to said human.
7. The method of claim 6, wherein said viral vector is an adeno-associated viral vector.
8. The method of claim 5, wherein said Factor IX is delivered to said mammal using an adeno-associated virus vector.
9. The claim 1, wherein said immunosuppressive agent is selected from the group consisting of cyclophosphamide, FK506, anti-CD40 ligand, CTLA4Ig, cyclosporin, antiB71-B72, and an immunosuppressive steroid.
10. The method of claim 9, wherein said immunosuppressive agent is cyclophosphamide.

11. The method of claim 9, wherein said immunosuppressive agent is FK506.

12. The method of claim 1, wherein said mammal has hemophilia B and said inhibitory antibodies specifically bind with Factor IX protein.

ABSTRACT OF THE INVENTION

The invention provides a method of preventing the formation of inhibitory antibodies in a mammal undergoing gene therapy. The method comprises administering to the mammal an immunosuppressive agent in conjunction with the
5 gene therapy.

Actions of Immunosuppressant agents

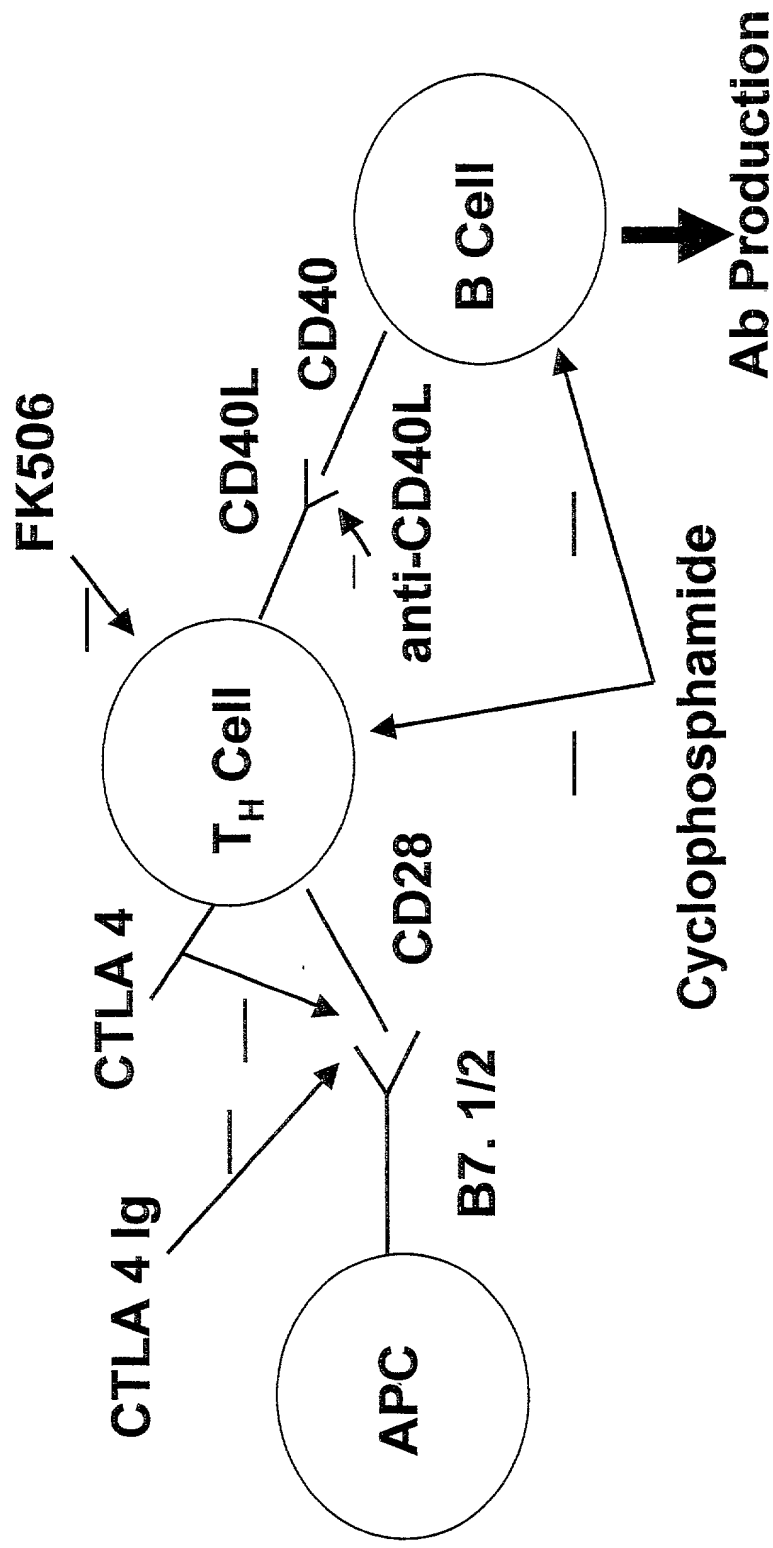


FIGURE 1

CD40 L antibody treated mice

Figure 2A

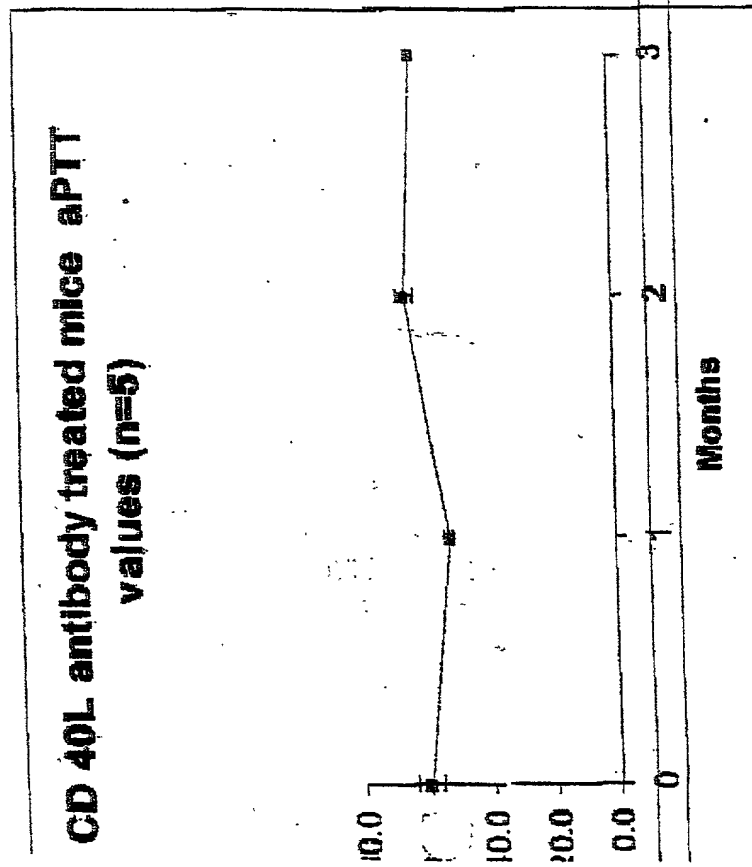


Figure 2B

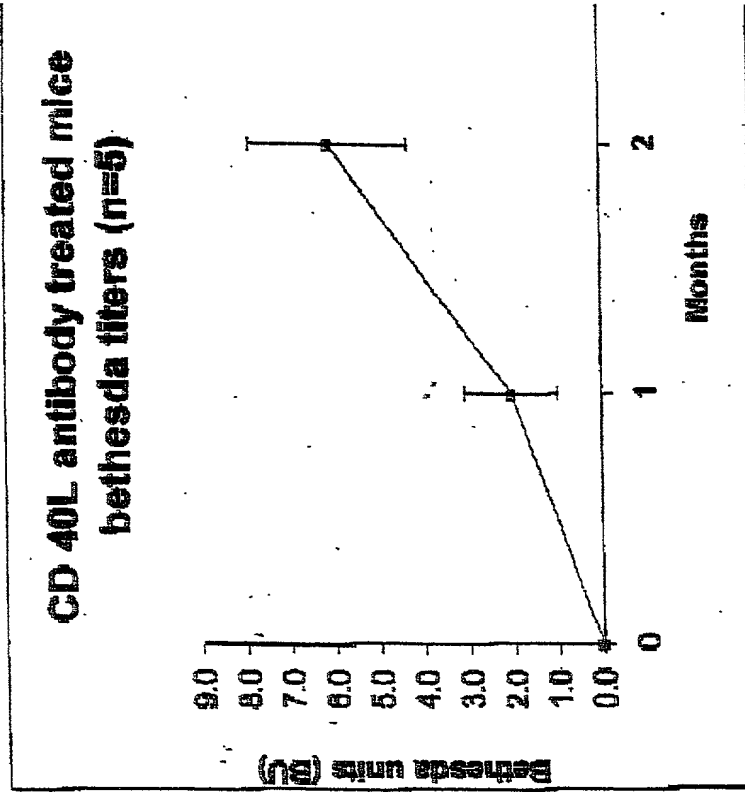


FIGURE 2

Cyclophosphamide treated mice

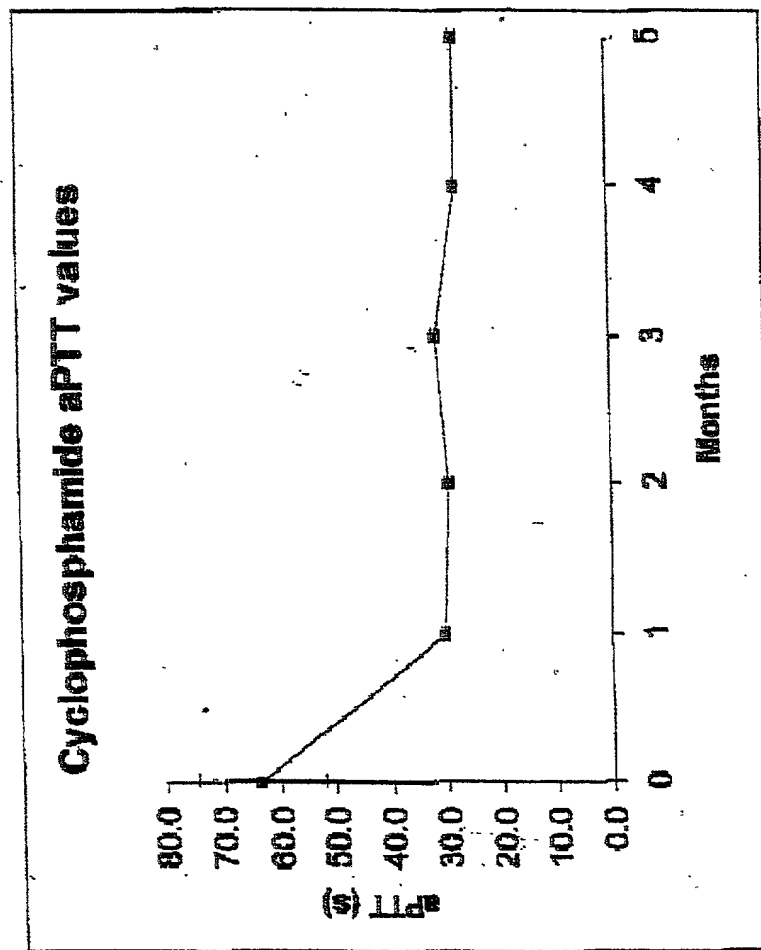


FIGURE 3

Anti-mF.IX in Hemophilia B Mice after IM injection with AAV-mF.IX

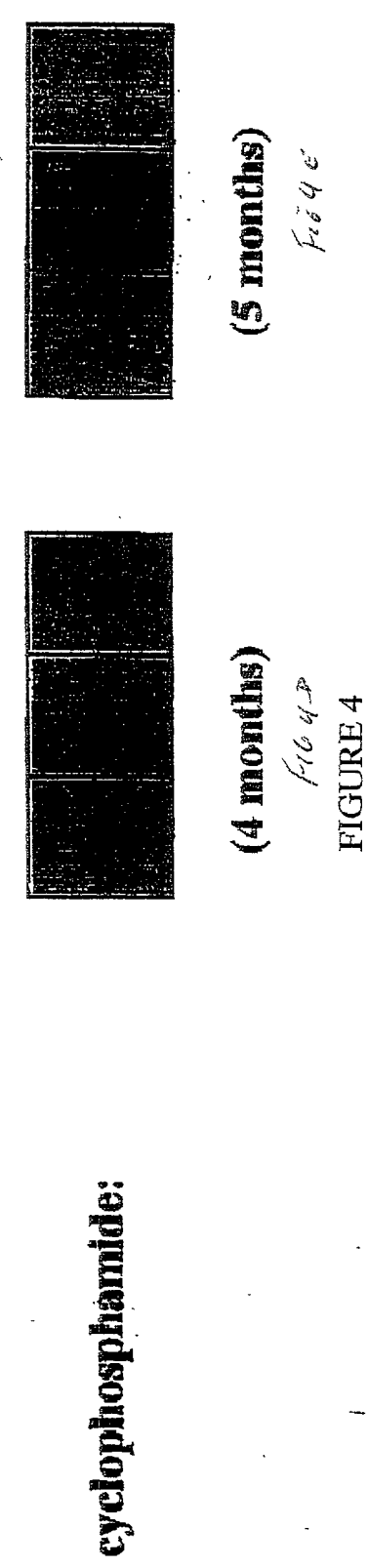
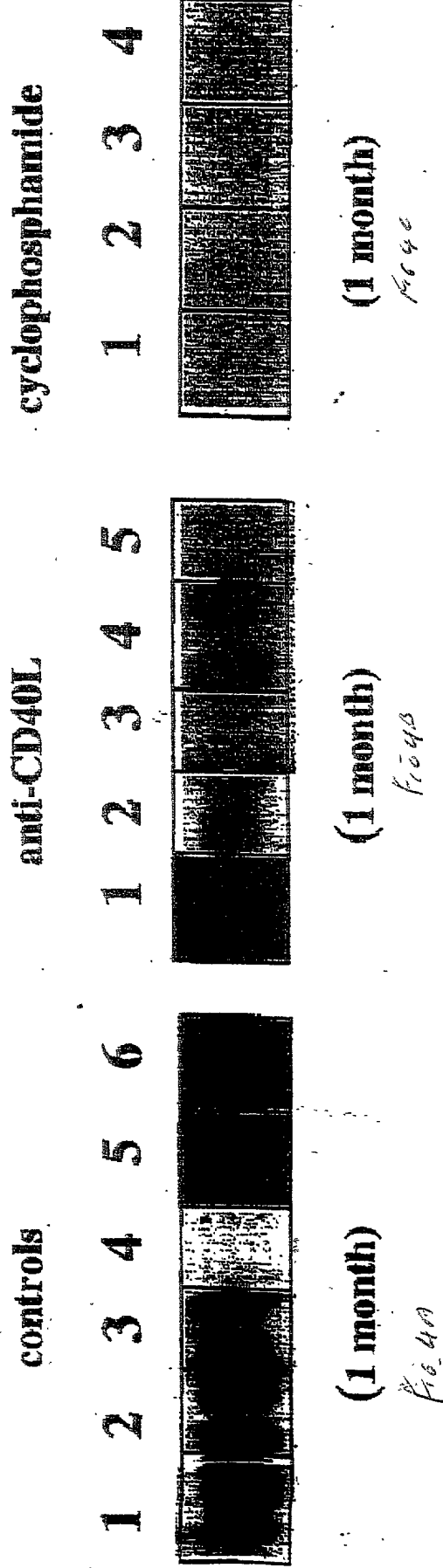


FIGURE 4

FK506 treated mice at 1 month

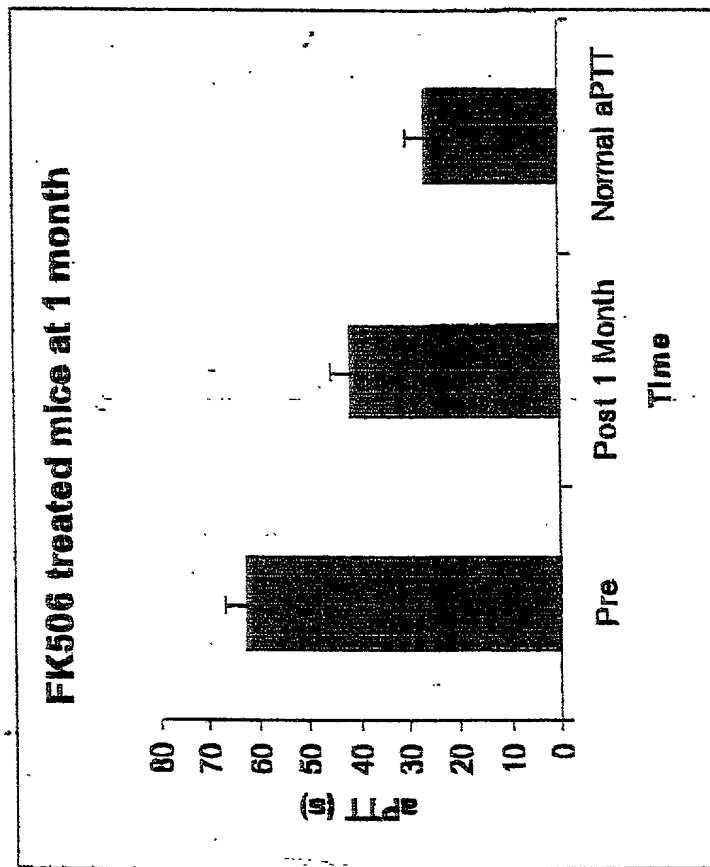


FIGURE 5

DECLARATION AND POWER OF ATTORNEY
(Related Application)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**METHODS FOR PREVENTING FORMATION OF INHIBITORY ANTIBODIES IN
THE SETTING OF GENE THERAPY**

the specification of which is attached hereto and/or was filed on **June 8, 2000** as Application No.: _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d), of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

FOREIGN PRIORITY APPLICATION(S)

Priority Claimed

☐ Yes ☐ No

(Number) (Country) (Day/month/year filed)

Priority Claimed

☐ Yes ☐ No

(Number) (Country) (Day/month/year field)

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional patent application(s) listed below and have also identified below any United States provisional patent application(s) having a filing date before that of the application on which priority is claimed.

PROVISIONAL PRIORITY PATENT APPLICATION

		<u>Priority Claimed</u>
<u>60/138,066</u>	<u>June 8, 1999</u>	<input checked="" type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Filing Date)	
 	 	<input type="checkbox"/> Yes <input type="checkbox"/> No
(Application No.)	(Filing Date)	

I hereby claim the benefit under Title 35, United States Code, Section 120, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or in the prior U.S. provisional application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status)--(patented, pending, abandoned)
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(Application Serial No.)	(Filing Date)	(Status)--(patented, pending, abandoned)
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And I hereby appoint the registered attorneys and agents associated with **AKIN, GUMP, STRAUSS, HAUER & FELD, L.L.P., Customer No. 000570**, as my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to **Customer No. 000570, namely, AKIN, GUMP, STRAUSS, HAUER & FELD, L.L.P.**, One Commerce Square, 2005 Market Street, Suite 2200,

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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